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MicroRNA-216a promotes M1 macrophages polarization and atherosclerosis progression by activating telomerase *via* the Smad3/NF-κB pathway☆

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ABSTRACT

Macrophages exhibit heterogeneity and plasticity and imbalance between pro-inflammatory and anti-inflammatory macrophages plays a critical role in atherosclerosis progression. Telomerase reverse transcriptase (TERT) in macrophages can be activated by nuclear factor-kappa B (NF-κB), but the regulation of telomerase activation on macrophages polarization remains unknown. We previously identified microRNA-216a (miR-216a) to promote inflammation through directly targeting the Smad3/NF-κB pathway. The present study aimed to assess whether miR-216a can regulate telomerase activity and promote macrophages polarization during atherosclerosis progression. The results verified that TERT was highly expressed in macrophages of human carotid atherosclerotic plaques. miR-216a was found to promote telomerase activation in macrophages by 4.5-fold ($P = 0.002$) through the Smad3/NF-κB pathway. miR-216a also induced macrophages senescence characterized by senescence-associated-β-galactosidase activity and p53 and p16 expression. TERT overexpression promoted the transformation of M2 to M1 while this conversion was suppressed once TERT was inhibited, and the related inflammatory factors and lipid uptake ability of M1 cells were also increased by TERT. In the carotid atherosclerotic plaques from miR-216a-treated apolipoprotein $E^{-/-}$ mice, the numbers of M1 macrophages were increased whereas M2 cells reduced, accompanying with inhibited Smad3 expression and upregulated inflammatory markers and TERT activity. Furthermore, plasma miR-216a level was specifically higher in patients with vulnerable mixed plaques ($n = 181$) than those with calcified plaques ($n = 73$) and controls ($n = 264$). In summary, our findings first revealed a new molecular mechanism of macrophage polarization involving telomerase activation induced by miR-216a through the Smad3/NF-κB signaling, which might serve as a potential therapeutic target for atherosclerosis progression.

1. Introduction

Atherosclerosis is an age-related, chronic inflammatory disorder and leads to high morbidity and mortality of cardiovascular diseases such as myocardial infarction and stroke. The monocyte/macrophages have been well-recognized as the central participant in the inflammatory process during atherosclerosis development, from initiation of lesions and lesion expansion to necrosis and eventually the rupture of plaques [1]. Macrophages display phenotypic heterogeneity and plasticity in atherosclerotic plaques, mainly including two kinds of subsets: M1 pro-inflammatory subtype and M2 anti-inflammatory subtype, which can be converted to each other under certain conditions and therefore

Abbreviations: ApoE, apolipoprotein E; CCTA, coronary computed tomography angiography; Col1a1, collagen type I alpha 1 chain; Col3a1, collagen type III alpha 1 chain; miRNA, microRNA; miR-216a, microRNA-216a; MCP1, monocyte chemotactic protein 1; Nos2, nitric oxide synthase 2; ox-LDL, oxidized low-density lipoprotein; SA-β-gal, senescence-associated-β-galactosidase; TERT, telomerase reverse transcriptase; THP-1, human acute monocytic leukemia cell line; TRAP, telomerase repeat amplification protocol

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contribute to the plaque progression [2, 3]. A better understanding of the pathogenesis of atherosclerotic plaque stability and searching for novel therapeutic targets are great challenges to medical society.

Emerging evidence have shown that the human advanced atherosclerotic plaques are rich with senescent endothelial cells and vascular smooth muscle cells characterized by elevated activity of senescence-associated-β-galactosidase (SA-β-gal) and gene expression of p16 and p53 [4, 5]. Recently, macrophages with senescence markers have also been found to accumulate within the plaque lesions and promote the inflammatory cytokines expression and metalloprotease production [6]. These findings indicate that vascular senescent cells are important drivers of atherosclerosis progression. Our previous work has identified a particular microRNA (miR-216a) which is mainly expressed in senescent endothelial cells and activated macrophages [7]. We observed that miR-216a can promote endothelial inflammation and senescence by directly targeting SMAD family member 3 (Smad3) and thereafter modulating downstream nuclear factor-kappa B (NF-κB) signaling. Gong et al. reported that miR-216a can regulate the ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux by targeting cystathionine γ-lyase in macrophage foam cells [8]. However, the regulatory role of miR-216a in macrophages differentiation and polarization during the plaque progression remains unknown.

Telomerase is a critical factor in regulating tissue renewal and involved in atherosclerosis and cardiovascular diseases [9]. Recent studies reported that the catalytic subunit telomerase reverse transcriptase (TERT) expression is remarkably increased in neutrophils isolated from coronary artery plaque of patients [10] and also in macrophages within the shoulder region of human advanced atherosclerotic plaques [11]. Gizard et al. demonstrated that genetic deficiency of TERT in macrophages can induce a phenotype of cellular senescence, on the other hand, telomerase in macrophages can be activated during the atherosclerosis formation in an LDL-receptor-deficient mice model, indicating a potentially important role of inducible TERT expression during inflammation [11]. Functional experiments further identified a conserved NF-κB response element in the TERT promoter and thus NF-κB can physically increase TERT transcription level in macrophages under inflammatory stimuli [11]. Since TERT is a potential target of NF-κB and our previous work showed that the Smad3/NF-κB signaling, regulated by miR-216a, is mainly responsible for the inflammatory process, we proposed the hypothesis that miR-216a might play a role in the senescence, differentiation and polarization of macrophages by regulating telomerase activity *via* Smad3/NF-κB pathway during the atherosclerosis progression.

In the current study, we investigated the distribution of TERT expression in human carotid atherosclerotic plaque lesions, measured the senescence-related markers in macrophages induced by miR-216a, and clarified the molecular mechanism of which miR-216a regulates macrophages polarization through telomerase activity during inflammatory remodeling and atherosclerotic plaque progression *in vitro* and in apolipoprotein *E*-deficient ($ApoE^{-/-}$) mice model. Furthermore, we established a coronary computed tomography angiography (CCTA) cohort to assess the relationship between plasma miR-216a level and the stability of plaques.

2. Materials and methods

2.1. Cell culture

The human acute monocytic leukemia cell line, THP-1 cells were cultured in RPMI Medium Modified with 10% fetal bovine serum (FBS) and then differentiated to M0 macrophages with 100 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 h. M0 macrophages were activated to M1 phenotype with 1 ng/ml lipopolysaccharide (LPS) and 20 ng/ml interferon (IFN)-γ for 24 h, or to M2 phenotype with 20 ng/ml interleukin (IL) 4. To establish stable cell line of TERT gene, THP-1 cells were infected with TERT recombinant lentiviruses or control vectors (LV201 lentiviral transfer vectors). Four days after infection, the cells were selected by enhanced green fluorescent protein (EGFP) florescence with the FACS Aria 2 Flow Cytometry/Cell Sorting System (BD Biosciences, San Jose, CA, USA). To assess the regulatory role of miR-216a on telomerase activity in macrophages, miR-216a mimics or miR-216a inhibitor were transfected at a final concentration of 50 nM with the use of lipofectamine 3000 reagent. After incubation for 48 h, the cells were harvested for further experiments. To assess the effect of telomerase on macrophages function, TERT siRNA was designed as the following: sense 5′ GGCCGAUUGUGAACAUGGAtt 3′; antisense: 5′ UCCAUGUUCACAAUCGGCCgc 3′. To assess the effect of Smad3 on telomerase activity in macrophages, Smad3 siRNA was designed and synthesized as following: sense 5′ AGGACGAGGUCUGCGUGAAUCC CUA 3′; antisense 5′ UAGGGAUUCACGCAGACCUCGUCCU 3′. The details were described in the online Supplementary data.

2.2. Immunofluorescent analysis for macrophages

To detect the effects of miR-216a on the expression and localization of telomerase and NF-κB in macrophages, the immunofluorescent tests of TERT and p65 were performed. The details were described in the online Supplementary data.

2.3. Human carotid atherosclerotic plaques

The human carotid atherosclerotic plaques ($n = 5$) and the nearby segments with normal carotid artery were obtained from patients undergoing carotid endarterectomy. The utilization of human vascular tissues was approved by the ethics committee of FuWai Hospital and all patients provided their written informed consents. The immunohistochemical and immunofluorescent tests were performed to determine the number of macrophages and telomerase expression in plaque lesions. The Picro Sirius Red staining was performed to detect the collagen content of carotid plaques. The details were described in the online Supplementary data.

2.4. Animal models with atherosclerotic plaque and histological analysis

An $ApoE^{-/-}$ mouse model of carotid atherosclerosis was established according to the method of Chen et al. [12], which exhibits unstable atherosclerotic lesions resembling characteristics of human vulnerable, rupture-prone plaques in carotid arteries. The male $ApoE-/-$ mice on a C57BL/6J background were fed Western Diet for 6 weeks, and at 12 weeks of age, were intraperitoneal injected the mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) for anaesthetization. Then, a surgery was conducted to introduce a tandem stenosis with 160 μm outer diameters on the right carotid artery bifurcation of $ApoE^{-/-}$ mice. The stenosis diameter was created by placing a 6–0 black, braided polyester fiber suture around the right carotid artery which was together tied a 160 μm needle and later removed. Local infiltration with pre-miR-216a recombinant lentiviruses (Ubi-EGFP-MCS-IRES-puromycin) or negative control vectors was given during the surgery. The mice were euthanized by 1% Pentobarbital sodium salt (50 mg/kg) after a high-fat diet for further 4 weeks, and the carotid vessel segments II in this tandem stenosis model were collected to analyze the effects of miR-216a on atherosclerosis development, $n = 10$ for each group. For histological analysis of plaque features, the 10 μm thick transversal cryosections were stained with standard Mayer's hematoxylin/eosin (HE), Oil red O to detect lipids, Masson to detect fiber, and Picro Sirius Red to detect collagen. To assess the number of different subtype macrophages, histological sections were incubated with primary antibodies anti-CD16 + CD32 (M1 marker) or anti-Arginase 1 (M2 marker). The details were described in the online Supplementary data.

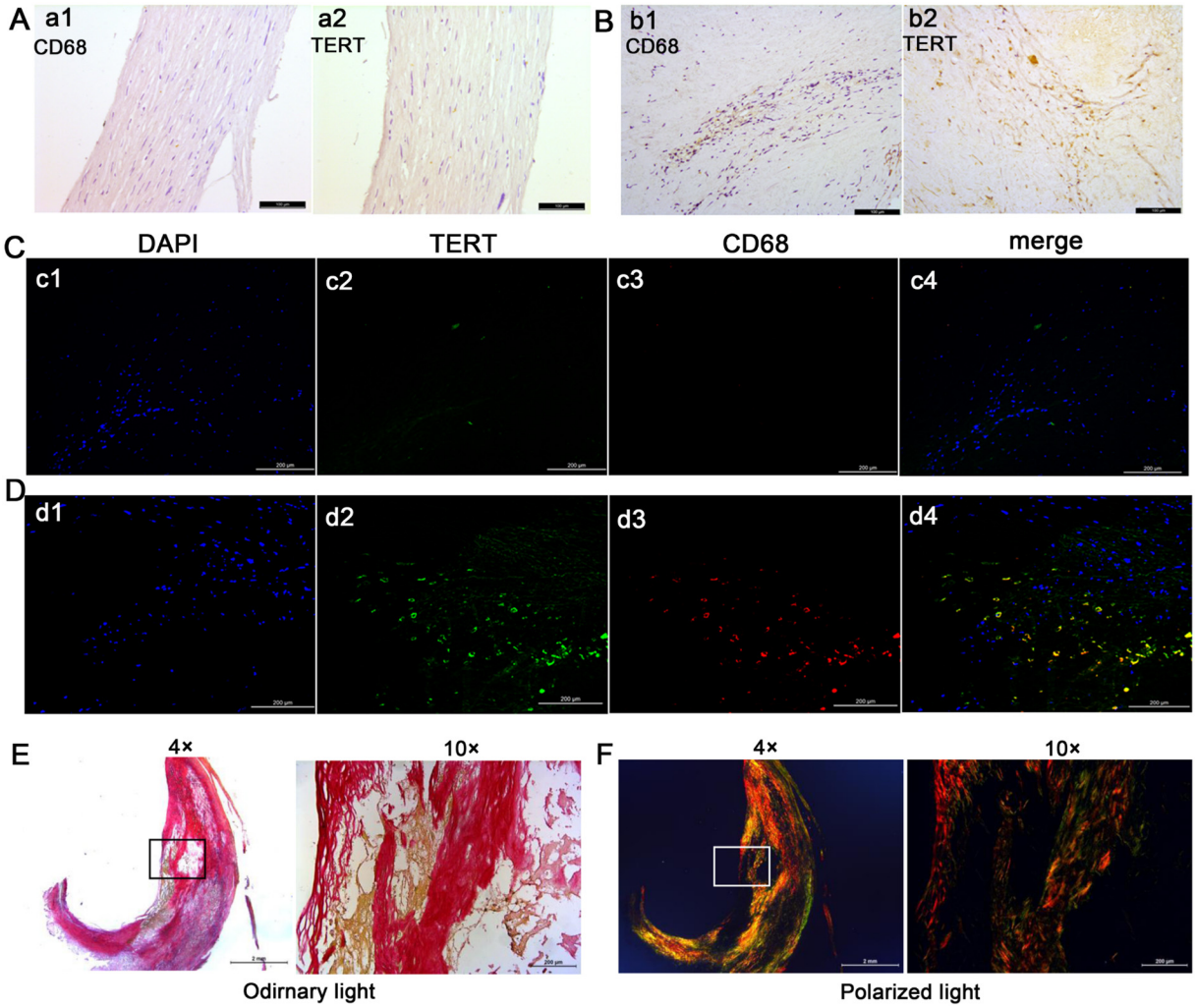

Fig. 1. Telomerase activity is increased in macrophages of human carotid artery atherosclerotic plaques.
A, B, Immunohistochemical staining of macrophages CD68 (a1, b1) and Telomerase reverse transcriptase (TERT) (a2, b2) in normal carotid arteries and carotid plaques obtained from patients undergoing carotid endarterectomy, scale bar = 100 μm, $n = 5$.
C, D, Representative immunofluorescence photographs of normal carotid arteries and lesions showed the colocalization of CD68 and TERT immunoreactivity. DAPI to visualize nucleus (blue, c1, d1), TERT (green, c2, d2), macrophages stained for CD68 (red, c3, d3) and the merged pictures showed colocalization of CD68 and TERT (yellow staining, c4, d4), scale bar = 200 μm, $n = 5$.
E, F, The collagen showed red color under ordinary light, and collagen I and collagen III were respectively indicated as red and green under polarized light determined by Picro Sirius Red staining. Scale bar = 2 mm (4×) or 200 μm (10×), $n = 5$.

2.5. The phagocytosis and lipid uptake abilities of macrophages

To investigate the role of miR-216a on the phagocytosis ability of THP-1 derived macrophages, the neutral red phagocytic experiment and acid phosphatase activity detection assay were performed. To explore the effects of miR-216a on lipid uptake ability of M1 macrophages, the foam cells formation assay was used by incubation of 50 μg/ml oxidized low-density lipoprotein (ox-LDL) and Oil red O staining. The details were described in the online Supplementary data.

2.6. SA-β-gal staining

M1 macrophages were transfected with miR-216a mimics for 48 h, and then the senescent status of macrophages was identified by *in situ* SA-β-gal staining. The details were described in the online Supplementary data.

2.7. Real-time PCR assay and western blot analysis

The mRNAs and miRNAs expression were analyzed by real-time PCR on the ABI 7500 System (Applied Biosystems, Foster City, CA, USA). The effects of miR-216a on Smad3 and NF-κB inhibitor alpha (IκBα) protein levels in macrophages were determined by western blot assay. The details were shown in the online Supplementary data.

2.8. Telomerase enzyme activity measurement

The telomerase repeat amplification protocol (TRAP) assay was performed to assess the telomerase activity during macrophages differentiation and polarization [13], with details shown in the online Supplementary data.

2.9. Patients recruitment and coronary artery image analysis

A total of 518 patients who were suspected with coronary artery disease and underwent CCTA at FuWai Hospital were consecutively recruited in the present study between May 2016 and October 2017. The study was approved by the ethics committee of FuWai Hospital and all subjects provided their written informed consents. CT scans were performed using a 64-row spiral CT scanner (Light Speed VCT, GE

Healthcare, Milwaukee, WI). All participants were divided into three groups, including control subjects without plaque ($n = 264$), patients with calcific plaques ($n = 73$), and patients with mixed plaques ($n = 181$, having components of both calcified and noncalcified plaques). An atherosclerotic plaque was identified with a structure > 1 mm^2 and clearly distinguished from the coronary artery lumen [14]. Calcium was defined as the presence of at least 3 contiguous pixels with a density > 130 HU and the plaques which composed exclusively of high density material > 130 HU were classified as the calcified plaque group [15]. Atherosclerotic lesions which consist of lipid, fibrous and calcified components were regarded as the mixed plaque group [16]. The clinical characteristics of participants were described in Table A.2 in the online Supplementary data.

2.10. Statistical analysis

Quantitative variables were presented as means ± S.D. or medians and interquartile ranges (IQRs), and categorical variables as percentages. The $\chi 2$ test was used to compare qualitative variables. Group differences of quantitative variables were compared by Student *t*-test, or One-way ANOVA, or the Mann-Whitney nonparametric test, as appropriate. Data were analyzed by SPSS Statistics 20.0 (SPSS Inc., Chicago, USA), and $P < 0.05$ was considered to be significant.

3. Results

3.1. Telomerase activity is increased in macrophages of human carotid artery atherosclerotic plaques

To test whether telomerase was activated in human atherosclerotic lesions *in vivo*, immunohistochemical analysis was performed on the carotid atherosclerotic plaques and the normal carotid artery segments, obtained from patients undergoing carotid endarterectomy surgery. The results showed that TERT expression was almost negligible in normal human carotid artery segments (Fig. 1A) but highly expressed in the macrophage-rich region of advanced atherosclerotic plaque lesions (Fig. 1B). The immunofluorescent test further showed that TERT was co-localized with CD68+ macrophage immunoreactivity (Fig. 1C and D). Next, the histological analysis showed that the human carotid artery plaques exhibited large plaque areas, big lipid cores and thin fibrous caps with collagen degradation by Picro Sirius Red staining test (Fig. 1E and F), indicating an advanced and unstable status of the plaques.

3.2. miR-216a promotes telomerase activity during macrophages differentiation

During macrophages differentiation, mRNA expression of TERT gene was upregulated by 1.8-fold ($P = 0.003$) and telomerase activity was increased by 8.3-fold ($P < 0.001$) in THP-1 derived macrophages (Fig. 2A). miR-216a expression was upregulated by 2.1-fold ($P = 0.001$) (Fig. 2B), paralleling with the increased expression of TERT. To further examine the role of miR-216a in telomerase activation during macrophages differentiation, THP-1 derived macrophages were transfected with miR-216a mimics or inhibitor. As shown in Fig. 2C and D, miR-216a overexpression markedly increased TERT expression by 30% ($P = 0.005$) and telomerase activity by 4.5-fold ($P = 0.002$), while miR-216a inhibition led to a decrease of TERT expression by 80% ($P = 0.001$) and telomerase activity by 88% ($P = 0.001$). Immunofluorescent analysis further indicated that miR-216a upregulated TERT expression and telomerase activity during macrophages differentiation (Fig. 2E and F).

In addition, we found that overexpression of miR-216a increased inflammatory factors level including monocyte chemotactic protein 1 (MCP1), tumor necrosis factor α (TNFα) and IL1β, while inhibition of miR-216a downregulated these inflammatory factors (Fig. A.1A and B in the online Supplementary data). The lipid uptake ability of THP-1 derived macrophage was also increased shown by the neutral red phagocytic experiment and acid phosphatase activity detection assay (Fig. A.1C and D in the online Supplementary data).

3.3. miR-216a induces telomerase activation during macrophages differentiation via the Smad3/NF-κB pathway

Smad3 has been proven to be a direct target of miR-216a and exerts an anti-inflammatory role in endothelial cells in our previous study [7]. To verify the role of miR-216a in macrophages, miR-216a mimics or inhibitor were transfected. The results showed that miR-216a significantly decreased Smad3 protein levels by 37% ($P = 0.02$) and downstream IκBα protein expression by 48% ($P = 0.03$); while, once endogenous miR-216a was inhibited, Smad3 and IκBα expression were respectively upregulated by 28% ($P = 0.02$) and 25% ($P = 0.04$) (Fig. 3A). The immunofluorescent analysis demonstrated that miR-216a increased the nuclear translocation of NF-κB p65 subunit in macrophages (Fig. 3B).

To confirm whether telomerase activation in macrophages induced by miR-216a was directly mediated through the Smad3/NF-κB pathway, Smad3 siRNA was transfected. Silencing Smad3 resulted in the upregulation of TERT expression by 30% ($P = 0.04$) (Fig. 3C), accompanied with increased inflammatory factors expression of NF-κB pathway, including MCP1, TNFα and IL1β (Fig. A.2A in the online Supplementary data). Immunofluorescent analysis further demonstrated that silencing Smad3 promoted nuclear translocation of NF-κB p65 subunit and TERT expression in macrophages (Fig. 3D). Under the condition that endogenous Smad3 was downregulated by siRNA, miR-216a inhibition was shown to rescue the expression of Smad3 and IκBα protein levels, and thereafter inhibit TERT expression by 27% ($P = 0.005$) (Fig. 3E). The expression of inflammatory factors in NF-κB pathway were also decreased (Fig. A.2B in the online Supplementary data).

3.4. miR-216a-induced telomerase activation promotes M1 macrophages polarization and lipid uptake ability

To elucidate the role of telomerase activation induced by miR-216a in macrophages polarization, the monocyte/macrophages polarization model was established *in vitro* and specific markers for M1 pro-inflammatory subtype and M2 anti-inflammatory subtype were examined (Fig. A.3A in the online Supplementary data). During macrophages polarization, miR-216a and TERT expression were upregulated by ≈2.0-fold in M1 macrophages ($P < 0.05$) but no significant change in M2 cells (Fig. 4A). miR-216a mimics or inhibitor were transfected to M1 and M2 macrophages, and the results showed that miR-216a promoted endogenous TERT by 8.3-fold ($P = 0.04$) but not in M2 cells (Fig. 4B), while miR-216a inhibition was shown to repress the TERT expression in M2 cells (Fig. A.3B in the online Supplementary data). In addition, miR-216a was found to upregulate the expression of inflammatory markers MCP1 and TNFα in M1 cells (Fig. 4C). Similar results were obtained in the ox-LDL-induced macrophages polarization model in which miR-216a promoted MCP1 and TNFα expression respectively by 38% ($P = 0.01$) and 24% ($P = 0.02$), whereas inhibition of miR-216a decreased these inflammatory markers expression (Fig. A.3C in the online Supplementary data), indicating an important role of miR-216a in atherosclerosis-induced macrophages polarization.

Notably, miR-216a significantly upregulated gene expression of p53 and p16 by 53% ($P = 0.04$) and 50% ($P = 0.02$), as well as the activity of SA-β-gal by 44% ($P = 0.03$) in M1 macrophages (Fig. 4D), indicating a potential effect of miR-216a on macrophages senescence. In addition, the lipid uptake ability of M1 macrophages was enhanced by miR-216a overexpression, but decreased when inhibiting the endogenous miR-216a (Fig. 4E).

The stable cell line of TERT recombinant lentiviruses was established to examine the role of telomerase activity in phenotype switching

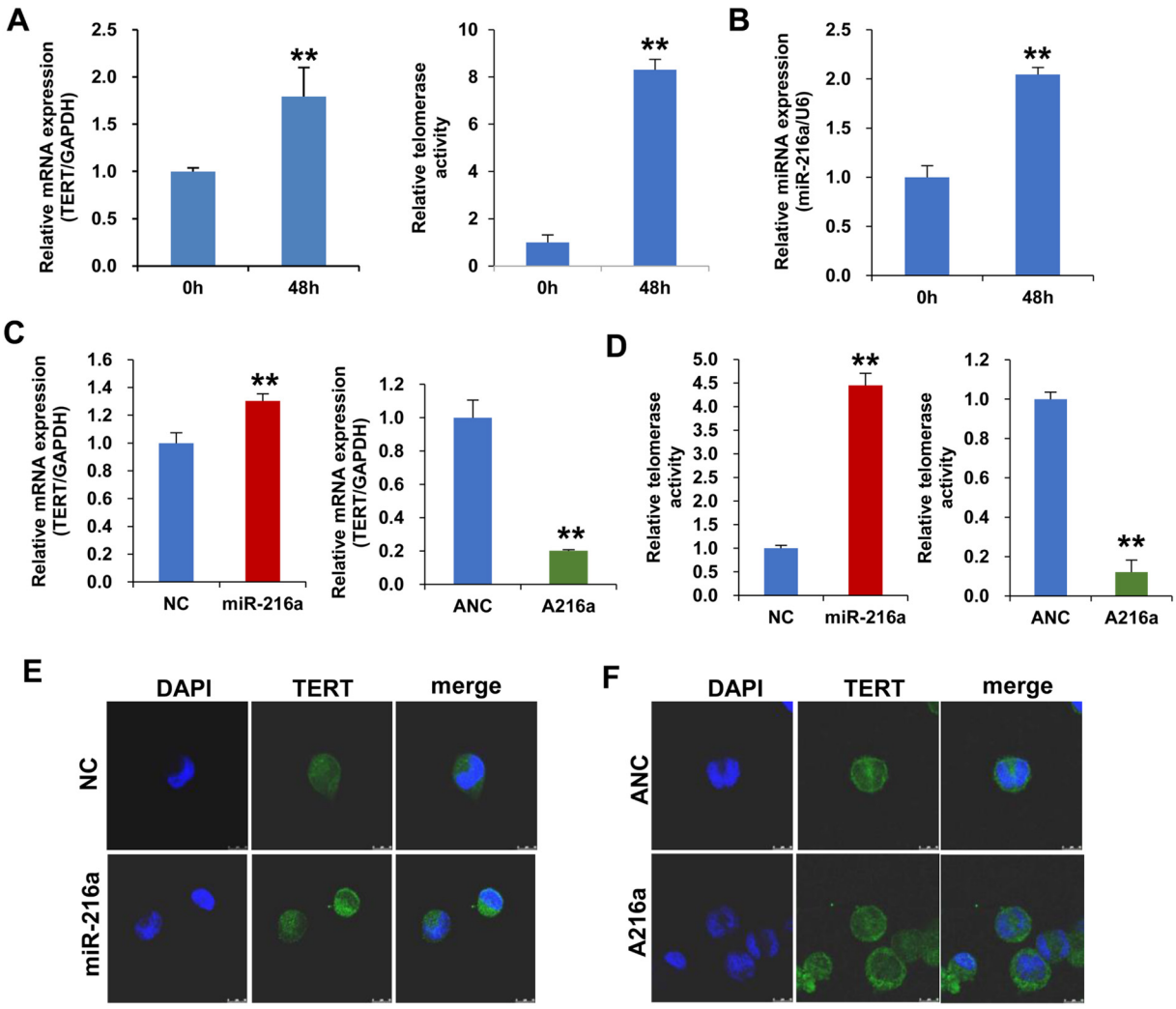

Fig. 2. miR-216a promotes telomerase activity during macrophages differentiation.
A, B, TERT mRNA expression, telomerase activity and mircoRNA-216a (miR-216a) expression during macrophages differentiation.
C, D, TERT mRNA expression and telomerase activity in THP-1 differentiated macrophages transfected with miR-216a mimics or inhibitor.
E, F, Representative immunofluorescent images staining for TERT (green) in THP-1 macrophages with miR-216a overexpression or inhibition. Scale bar = 10 μm.
$^{*}P < 0.05$, $^{**}P < 0.01$. Compared with negative control (NC) or inhibitor negative control (ANC). $N = 5$ for each group.

of M1 and M2 macrophages. The results showed that TERT overexpression promoted the transformation from M2 to M1 phenotype, accompanied with increased expression of M1 markers such as MCP1 and TNFα; on the contrary, when endogenous TERT was inhibited by its siRNA, the process of M2 to M1 conversion was repressed (Fig. 4F). During the foam cells formation induced by ox-LDL stimuli, overexpression of TERT accelerated the transformation from M1 macrophages to foam cells whereas knockdown of endogenous TERT with siRNA prevented the formation of foam cells (Fig. 4G). Under the condition that endogenous TERT activity was silenced by TERT siRNA, miR-216a overexpression was found to rescue the expression of TERT gene, thereafter promoting the ox-LDL-induced formation of M1 macrophages to foam cells and increasing the lipid uptake ability (Fig. 4H). All these results indicated that miR-216a-induced telomerase activation promoted macrophages polarization and phenotype transformation.

3.5. miR-216a promotes M1 macrophages polarization in carotid atherosclerotic plaques of mouse model

To elucidate the molecular mechanism of miR-216a on the regulation of Smad3/NF-κB pathway and telomerase in macrophages polarization and atherosclerosis development *in vivo*, an ApoE$^{-/-}$ mouse model of carotid atherosclerosis was established and the carotid vessel segments II with stenosis were collected (Fig. A.4 in the online Supplementary data). Consistent with *in vitro* data, we found that Smad3 expression was inhibited and TERT activity was upregulated in the carotid atherosclerotic plaques from miR-216a-treated mice (Fig. 5A). The inflammatory markers (MCP1, TNFα and IL1β) and matrix metallopeptidase 9 (MMP9) were increased, and the expression of nitric oxide synthase 2 (Nos2), known as M1 macrophages marker, was upregulated by 62% ($P = 0.04$) while the M2 marker IL10 was decreased by 52% ($P = 0.002$) in miR-216a-treated mice (Fig. 5B). The immunofluorescent staining of CD16 + CD32 (M1 marker) and Arginase 1 (M2 marker) further showed that M1 subsets were markedly increased whereas M2 subsets were decreased simultaneously, compared with plaque lesions from the control mouse model (Fig. 5C and D). Histological analysis of Oil red O staining showed no significant difference in lipid accumulation for the plaques between miR-216a-treated mice and control mice (Fig. A.5 in the online Supplementary data). However, miR-216a significantly downregulated the expression of collagen type III alpha 1 chain (Col3a1) by 66% ($P = 0.017$) and the amounts of collagen III shown by Picro Sirius Red staining in carotid atherosclerotic plaques, while the collagen type I alpha 1 chain (Col1a1) was not affected (Fig. 5E and F).

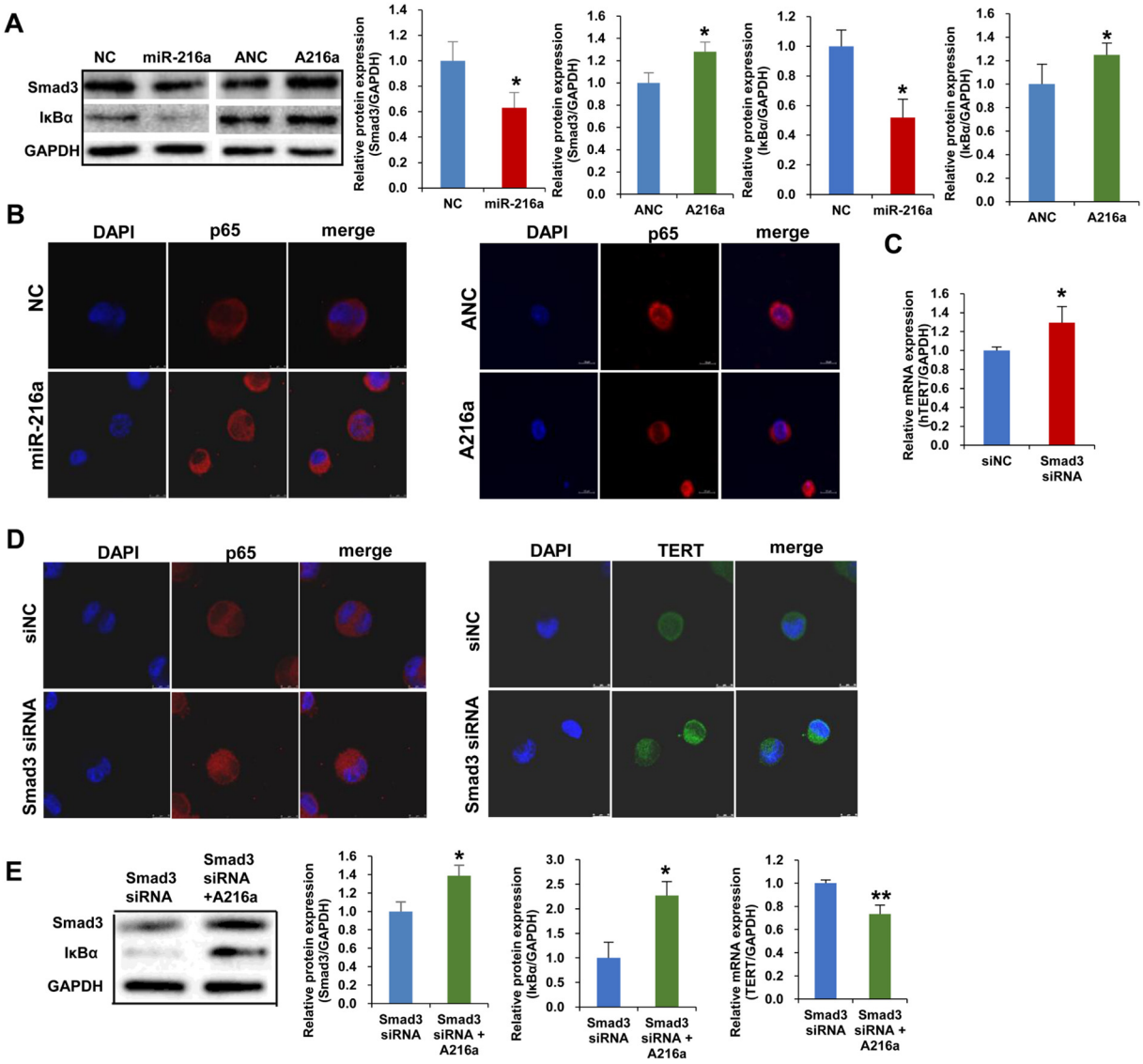

Fig. 3. miR-216a induces telomerase activation during macrophages differentiation *via* the Smad3/NF-κB pathway.
A, SMAD family member 3 (Smad3) and NF-κB inhibitor alpha (IκBα) protein levels in THP-1 derived macrophages transfected with miR-216a mimics or inhibitor.
B, Nuclear translocation of NF-κB subunit p65 induced by miR-216a using immunofluorescent assay (DAPI, blue; p65, red).
C, TERT mRNA expression in THP-1 derived macrophages induced by Smad3 siRNA.
D, Representative immunofluorescent images staining for p65 (red) and TERT (green) of macrophages transfected with miR-216a mimics or inhibitor.
E, The mRNA expression of TERT and the protein expression of Smad3 and IκBα in macrophages with simultaneous transfection of Smad3 siRNA and miR-216a inhibitor.
*$P < 0.05$, **$P < 0.01$. Scale bar = 10 μm. $N = 5$ for each group.

3.6. Plasma miR-216a level is elevated in patients with vulnerable coronary plaques

According to the features of coronary plaque images by CCTA examination, all subjects in this study were divided into three groups, including controls without plaque ($n = 264$), calcific plaque (stable plaques, $n = 73$), and mixed plaque (vulnerable plaque, $n = 181$). Plasma miR-216a level was significantly higher in the mixed plaque group (1.04, IQR 0.49–2.01) than in the calcific plaque group (0.79, IQR 0.43–1.47) ($P = 0.03$) and in the controls (0.72, IQR 0.26–1.47) ($P = 0.007$), respectively. There was no significant difference for plasma miR-216a between the calcific plaque group and the controls ($P = 0.54$) (Fig. 6).

4. Discussion

Macrophages in the atherosclerotic plaque can respond to the micro-environmental stimuli and exhibit the heterogeneity and plasticity, in which imbalance between pro-inflammatory and anti-inflammatory macrophages polarization plays a critical role in the progression of atherosclerotic plaque. In this study, we first provided the *in vitro* and *in vivo* evidence that miR-216a promoted macrophage differentiation toward the M1 pro-inflammatory phenotype and increased macrophage's lipid uptake ability by activating telomerase, which was directly mediated through the Smad3/NF-κB pathway. Furthermore, miR-216a upregulated the number of M1 macrophages while reduced M2 macrophages in the carotid atherosclerotic plaques from miR-216a-treated mice. Plasma miR-216a level was also found to be significantly higher in patients with the coronary mixed plaques than those with the calcific plaques and control subjects without coronary plaque. All these

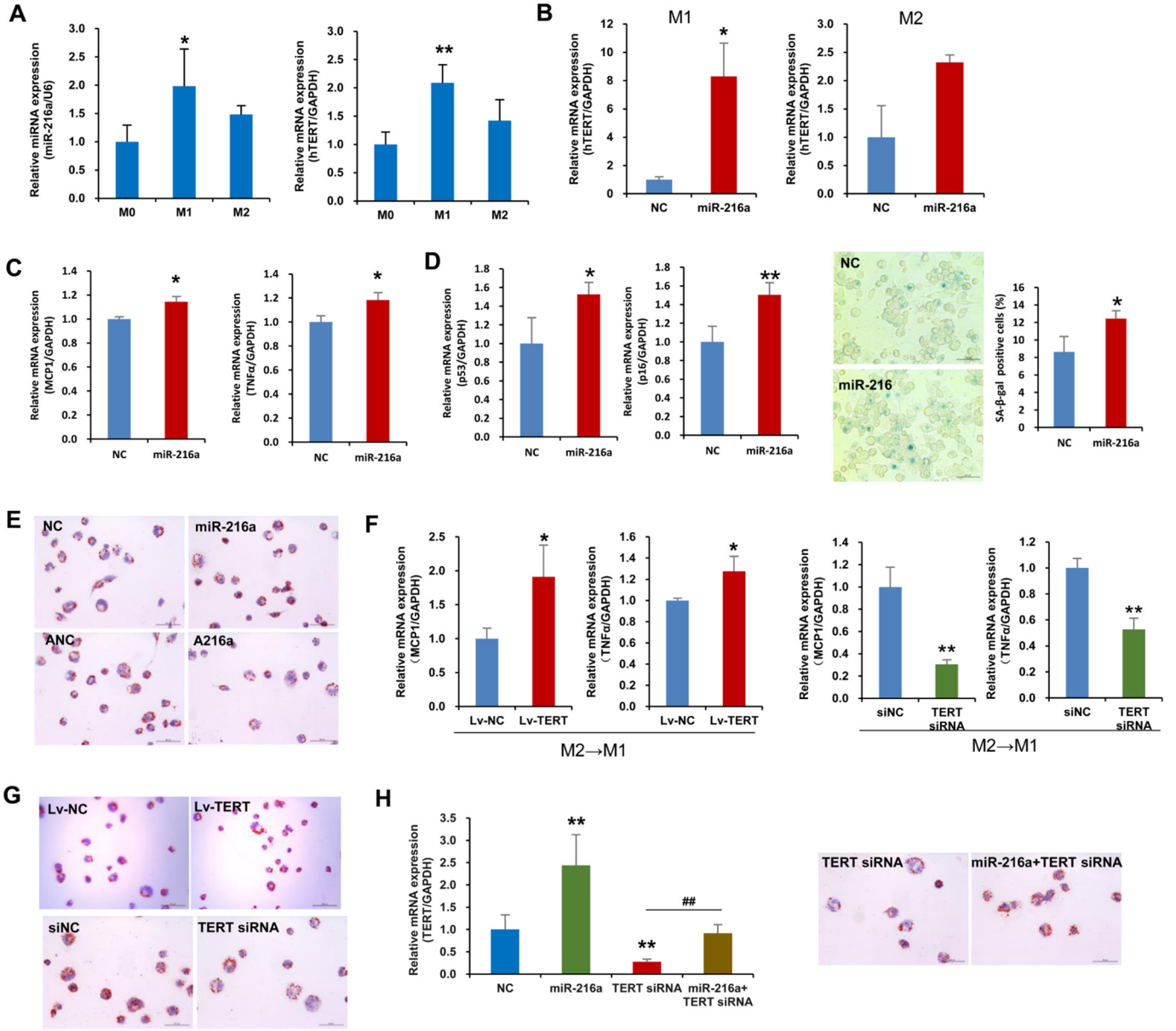

Fig. 4. miR-216a-induced telomerase activation promotes M1 macrophages polarization and lipid uptake ability.

A, miR-216a and TERT expression in interferon (IFN)-γ and lipopolysaccharide (LPS) induced M1 subtypes, interleukin (IL) 4 induced M2 macrophages and unpolarized macrophages M0.

B, TERT mRNA expression induced by miR-216a overexpression in M1 and M2 macrophages.

C, The mRNA expression of monocyte chemotactic protein 1 (MCP1) and tumor necrosis factor α (TNFα) in M1 macrophages.

D, p53 and p16 mRNA expression and the percentage of senescence-associated-β-galactosidase (SA-β-gal) positive cells in M1 macrophages transfected with miR-216a mimics for 48 h.

E, The lipid uptake ability of M1 macrophages induced by miR-216a, determined by incubation with 50 μg/ml oxidized low-density lipoprotein (ox-LDL) and Oil red O staining.

F, MCP1 and TNFα mRNA expression induced by lentiviral overexpression or siRNA knockdown of TERT during the transformation of M2 to M1.

G, The lipid phagocytic capability of M1 macrophages with lentiviral overexpression or knockdown of TERT.

H, TERT expression and lipid uptake ability in M1 polarized cells with simultaneous transfection of TERT siRNA and miR-216a mimics. * compared with negative control (NC), # compared with TERT siRNA.

$^{*}P < 0.05$, $^{**}P < 0.01$. Scale bar = 50 μm. $N = 5$ for each group.

findings proposed a new molecular mechanism of macrophage polarization involving telomerase activation induced by miR-216a, which might serve as a potential therapeutic target for macrophages polarization and atherosclerosis progression.

Telomerase exerts a critical role in preserving chromosome stability and regulating tissue renewal, and increasing evidence indicate that the catalytic subunit TERT can be activated in response to inflammatory stimulus [17]. In this study, we first demonstrated that miR-216a, located at chromosome 2p16.1, can promote telomerase activation and TERT expression in macrophages by directly targeting Smad3, thereafter modulating downstream NF-κB pathway. Several mechanisms may explain the activation of telomerase during macrophage inflammation. Firstly, TERT has been proven to be a potential target of NF-κB *via* a highly conserved NF-κB response element within the promoter region,

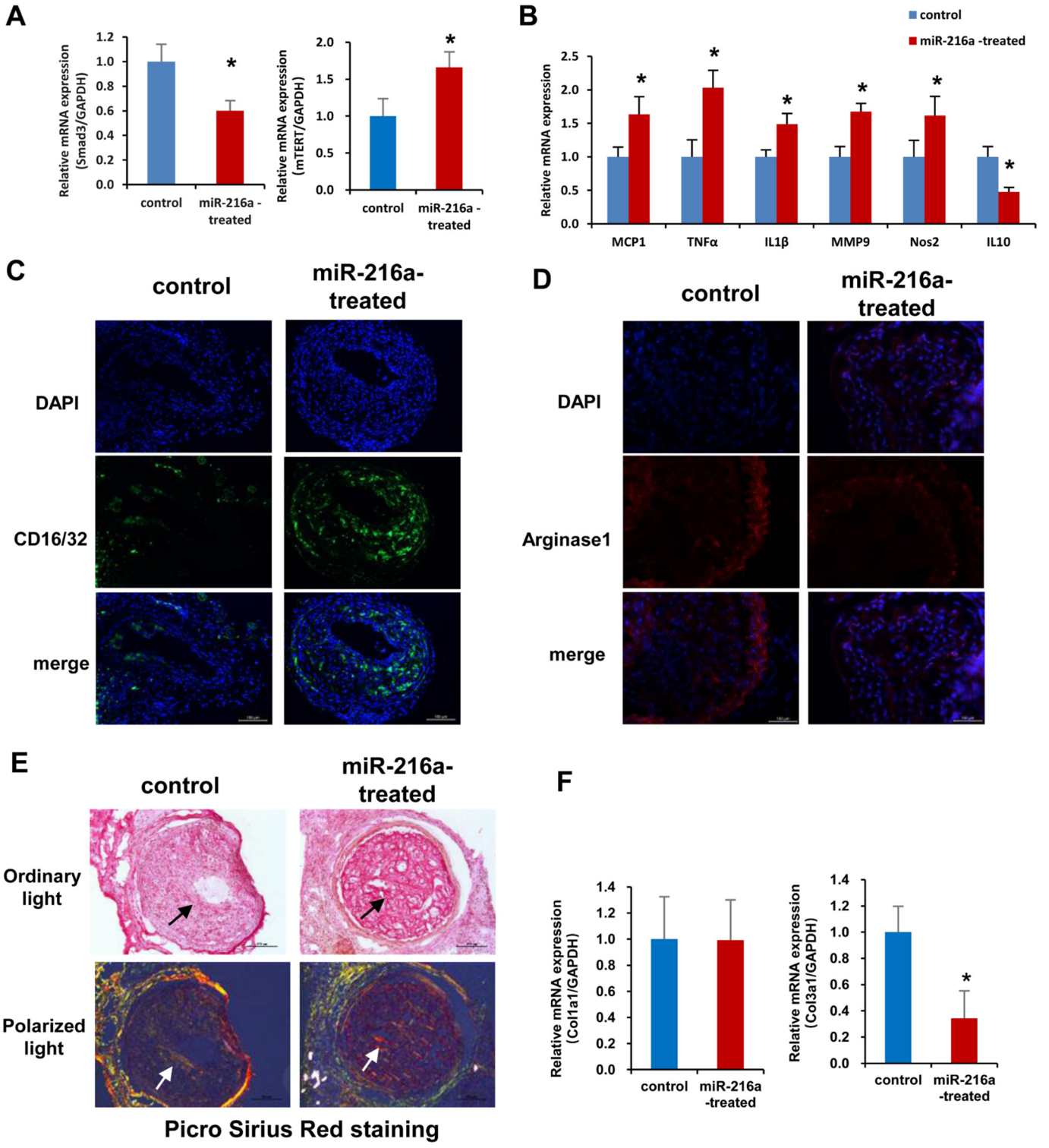

Fig. 5. miR-216a promotes M1 macrophages polarization in carotid atherosclerotic plaques of mouse model.
A, The mRNA expression of Smad3 and mTERT in carotid plaques induced by miR-216a or control.
B, The mRNA expression of MCP1, TNFα and IL1β, matrix metallopeptidase 9 (MMP9), M1 marker nitric oxide synthase 2 (Nos2) and M2 marker IL10 in carotid plaques of control or miR-216a group.
C, D, The immunofluorescent staining for M1 marker CD16 + CD32 and M2 marker Arginase 1 in carotid plaques of middle stenosis segment II.
E, Collagen I (red) and collagen III (green) in carotid lesions determined by Picro Sirius Red staining under polarized light.
F, Collagen type I alpha 1 chain (Col1a1) and collagen type III alpha 1 chain (Col3a1) mRNA expression in carotid plaques.
$^{*}P < 0.05$, $^{**}P < 0.01$. Compared with control. Scale bar = 100 μm. $N = 5$ for each group.

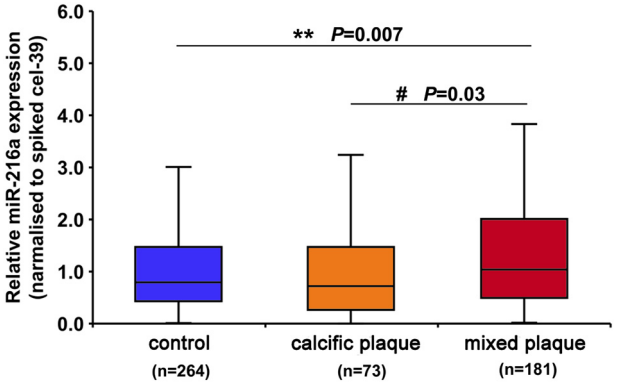

Fig. 6. Plasma miR-216a level is elevated in patients with vulnerable coronary plaques.

Plasma miR-216a levels were examined in 518 subjects who undergoing coronary computed tomography angiography (CCTA). All individuals (n = 518) were divided into three groups, including controls without plaque (n = 264), calcific plaque group (stable plaque, n = 73) and mixed plaque group (vulnerable plaque, n = 181). $^{**}P < 0.01$, compared with control. $\#P < 0.05$, compared with calcific plaque group.

thus, NF-κB can be rapidly recruited and then increased TERT transcription in macrophages [11]. Another potential mechanism is that NF-κB p65 can mediate TNFα-induced nuclear translocation of TERT protein *via* posttranslational mechanisms and therefore promote telomerase activity in macrophages [18].

We observed that telomerase activation induced by miR-216a can remarkably increase inflammatory factors expression, especially M1 macrophage associated markers such as MCP1, TNFα and IL1β, which may be implicated in an interaction of TERT and NF-κB pathway. Ghosh et al. [19] reported that TERT can regulate inflammatory signaling through directly binding to NF-κB promoter region and afterward activating the transcription of NF-κB-regulated genes such as TNFα and IL6. Wu et al. [20] further showed that TERT is in a feedback loop with NF-κB signaling pathway to regulate macrophage polarization in a mouse model of alcoholic liver disease. This evidence indicate that there exists a positive feed-back loop between telomerase activation induced by miR-216a and NF-κB pathway to promote M1 macrophages inflammation and polarization.

Our previous work showed that miR-216a is mainly expressed in senescent endothelial cells and promotes endothelial inflammation and senescence by Smad3/NF-κB signaling [7]. In this study, although TERT expression in macrophages was activated by miR-216a, we also found that miR-216a induced a senescent status of macrophages with elevated activity of SA-β-gal and gene expression of p16 and p53. It is known that limiting TERT expression can result in cellular senescence, and Gizard et al. showed that genetic deficiency of TERT gene in macrophages can lead to a senescent phenotype [11]. Considering that vascular senescent cells are important drivers of atherosclerosis progression, this paradoxical phenomenon may reflect the complex status of macrophages in the advanced atherosclerotic plaques. On one hand, transient TERT expression during inflammation may prevent senescence of the macrophages and potentially to maintain cellular responsiveness to immunity during atherosclerosis [11]. But on the other hand, macrophages with senescence markers accumulated within the plaque lesions are found to promote the inflammatory cytokines expression and metalloprotease production [6]. Thereafter, telomerase activity may be increased through the feed-back loop between TERT expression and NF-κB pathway mentioned earlier.

M1 macrophages are associated with symptomatic and unstable plaques, whereas M2 macrophages are particularly abundant in stable regions of the plaque as well as asymptomatic lesions [21]. In agreement with *in vitro* results, *in vivo* data from the advanced carotid atherosclerotic plaques of mouse model showed that miR-216a can increase the number of M1 macrophages whereas simultaneously reduce M2 cells, accompanying with the upregulated expression of TERT and inflammatory markers associated with unstable plaques, such as MCP1, TNFα, IL1β, and MMP9. Although no significant differences in plaque areas and lipid accumulation were observed between the miR-216a-treated mice and control mice, we found that miR-216a significantly decreased the amounts of collagen III and mRNA expression of Col3a1 in carotid plaques. Collagen type I is usually rich in plaque protein content and comprises mainly of thick fibers, whereas collagen type III comprises mainly of thinner fibers and promotes the fibrillogenesis of collagen type I. The loss of collagen type III can lead to a reduction in mature type I fibrils, which may promote plaque destabilization and weakening of the fibrous cap [22]. In support with our findings, Kong et al. reported that collagen type III expression is lower in plaques of patients with acute myocardial infarction [23]. These *in vivo* data indicated that miR-216a can promote the progression of atherosclerosis by inducing M1 macrophages polarization and inhibiting collagen III formation in the plaque lesions.

In this study, we verified that TERT is specifically expressed in the macrophage-rich region of human advanced atherosclerotic plaque lesions in carotid artery, whereas almost negligible in normal human carotid artery segments. These findings are consistent with previous studies by Matthews et al. [5] and Gizard et al. [11] who observed that TERT is highly expressed in macrophages within the shoulder region of atherosclerotic lesions in human coronary artery. Similarly, Narducci et al. has reported that TERT is remarkably increased in neutrophils isolated from coronary artery plaque of patients [10]. Moreover, we observed that plasma miR-216a level was specifically higher in patients with more vulnerable plaques than those with stable plaques and controls without plaques, suggesting that miR-216a may serve as a new potential marker for the prediction of plaques status. The prospective follow-up study is important to clarify the role of plasma miR-216a in predicting the future rupture and outcomes of atherosclerotic plaques.

5. Conclusions

In this study, we verified that TERT is highly expressed in macrophages of human advanced carotid atherosclerotic plaques. We for the first time showed that miR-216a can induce TERT activation in macrophages through the Smad3/NF-κB signaling, subsequently, promote M1 macrophages polarization and the lipid uptake ability. A senescent status of macrophages was also found to be induced by miR-216a, indicating the complex effects of miR-216a on macrophages function. Further data from the advanced carotid atherosclerotic plaques of mouse model showed that miR-216a can increase the number of M1 macrophages whereas reduce M2 cells, accompanying with the upregulated expression of TERT and inflammatory markers. Furthermore, plasma miR-216a level was elevated in patients with vulnerable coronary plaques, which indicating that miR-216a might serve as a potential therapeutic target for macrophages polarization and atherosclerosis progression.

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Authors' contributions

Zhang W conceived of, designed, and supervised the study. Yang S, Li J, Chen Y, Zhang S, Hou Z, and Feng C performed the experiments and analyzed the data. Cai J, Lv B and Hui R provided assistance with the experiments and data collection. Zhang W and Yang S wrote the manuscript. All authors have reviewed and approved this version of the manuscript.

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Conflict of interest

None.

Transparency document

The http://dx.doi.org/10.1016/j.bbadis.2018.06.016 associated with this article can be found, in online version.

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